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FOREWORD

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(5) INTRODUCTION

We hypothesized that components of DNA-related checkpoint pathways in addition to members of the *ATM* protein/lipid kinase family are conserved in all eukaryotes. This is based on functional similarities in the pathways and the conservation between the evolutionarily disparate budding and fission yeasts. Our goal is to identify additional regulators of mammalian DNA checkpoints, by virtue of structural and functional homology with known checkpoint genes in budding yeast. We proposed to use both structural and functional screens to identify human homologs of yeast damage checkpoint proteins Rad53 and Rad9. Once identified, such components will be ordered into pathways for mammalian checkpoint function, with emphasis on p53 regulation, cell cycle regulation, and complementation of *ATM* defects.

(6) BODY

We report here significant progress on Technical Objective 1, Technical Objective 3, and the ultimate goal of identifying mammalian checkpoint genes homologous to the checkpoint protein kinase Rad53.

1. Technical Objective 1. Protein-interaction screens for mammalian Rad53 and Rad9 homologs.

The overall objective of this aim is to use protein-based screens in order to identify mammalian homologs of yeast Rad53 and Rad9. In order to do this, the relevant protein interaction domains will first be identified using the yeast genes, and then the homologous sequences of identified mammalian genes (*ATM* and *CHK2/CDS1*) will be used as baits in protein interaction screens for novel mammalian partners.

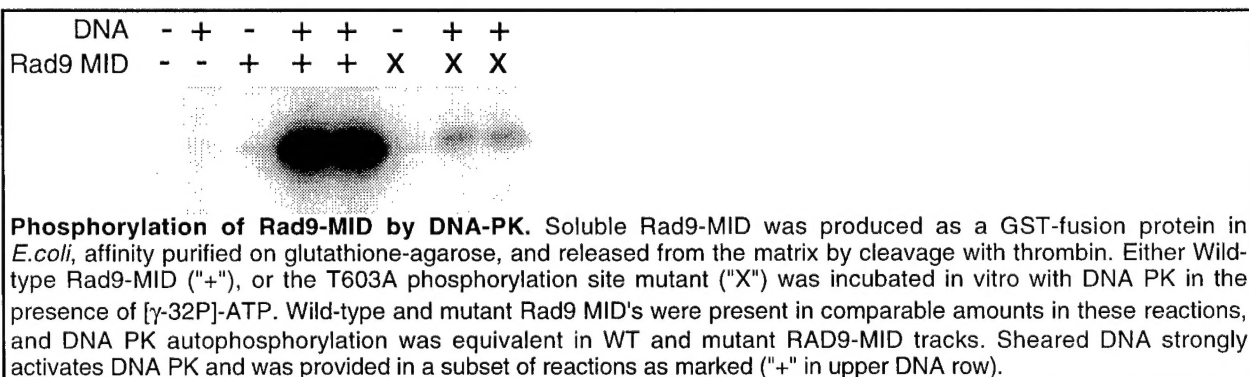
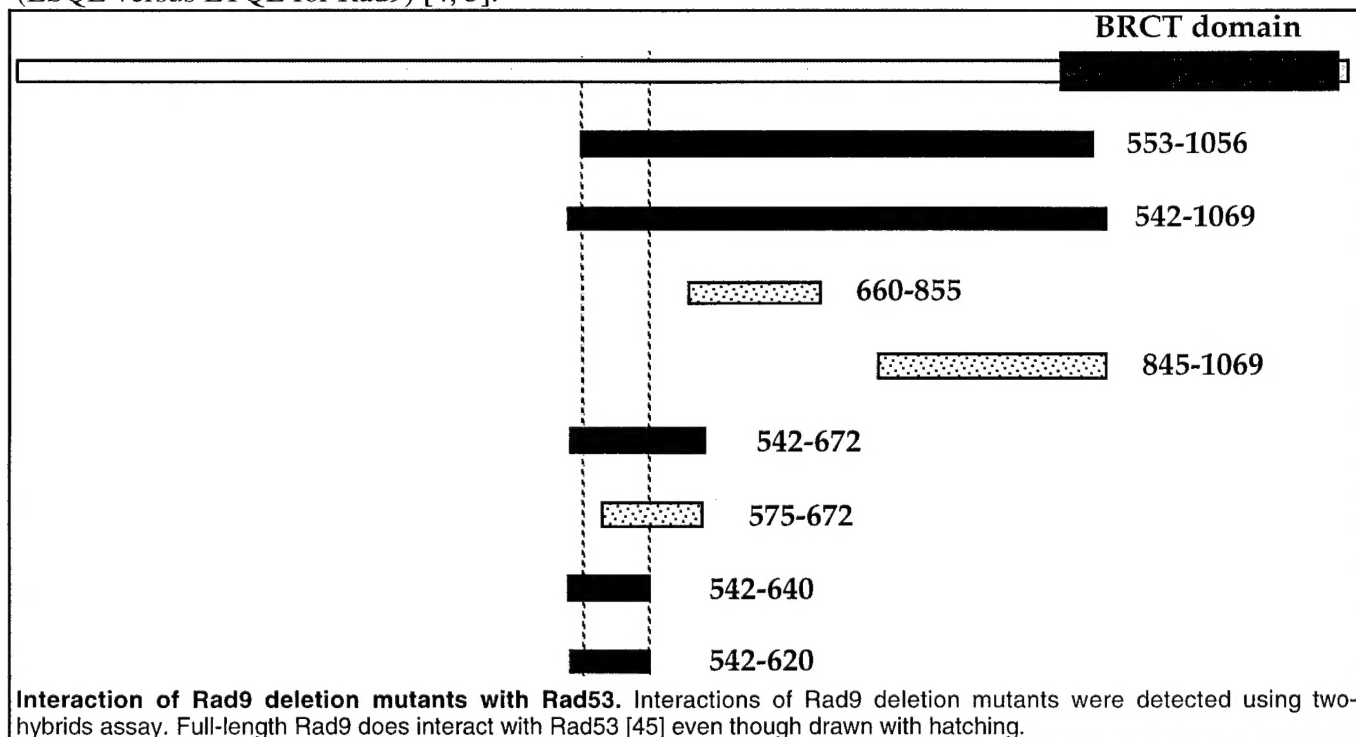
A. Tools for mapping interaction of Mec1 with Rad53 and Rad9. The first strategy was to identify a domain of (yeast) Mec1 that is responsible for interaction with Rad53 and/or Rad9, and then to use the homologous domain from (mammalian) Atm to identify dimerization partners. Since this aim first requires that we express functional Mec1 in order to map interactions with Rad53, we have endeavored to express this large gene in yeast and in mammalian cells. A *MEC1* gene was cloned from yeast by recombinational rescue, and moved into vectors for expression in mammalian cells, bacteria, and budding yeast. Efforts to express the polypeptide (marked with a V5 epitope tag) through transient transfection into COS-7 cells have thus far been unsuccessful. This is not completely surprising, since there is likely to be functional conservation of Mec1 and Atm, and Atm has proven to be difficult to express in mammalian cells. This is probably because it is a checkpoint gene, and has growth inhibitory properties. Ongoing efforts will be modeled after those that were ultimately successful with Atm, namely extraction of proteins from early time points, and careful evaluation of extraction conditions.

We have now successfully produced a portion of Mec1 (encompassing the putative kinase domain) as a Glutathione-S-Transferase (GST-) fusion protein in bacteria. This polypeptide is easily purified, and will be used along with other Mec1 deletion mutants expressed in bacteria for pull-down experiments to map the sites of interaction with Rad53 and Rad9, and as a source for immunogen for antibody production. As an alternative approach, we have produced yeast expression vectors for expression of V5-tagged MEC1, and these are now under control of both *GALI*- driven, and endogenous promoter- driven constructs. The former has been tested for the ability to complement *mec1* mutations, thus confirming the functionality of the clone. Preliminary data indicate that V5-expressed Mec1 is associated with protein kinase activity. Finally, we have introduced Mec1 into a two-hybrids screening vector, so that we can use two-hybrids screening to map interacting domains of Mec1 with Rad9 and Rad53.

B. Identification of Rad9/Rad53 interaction domain. Using a similar logic, we have endeavored to localize the domain on Rad9 that interacts with Rad53. We have already demonstrated that this interaction is a prototype for interactions of the FHA (*forkhead*-associated) protein homology domain with phospho-peptides [1]. By mapping the interaction domain with Rad9, we have now identified a candidate FHA target site. This information can then be used to

identify and evaluate candidate mammalian proteins that interact with the authentic Rad53 homolog.

The domain of Rad9 that binds to Rad53 contains a target site for Atm family protein kinases that is required for interaction with Rad53 [1]. Identification of the domain of Rad9 that interacts with Rad53 FHA2 is important for understanding this regulatory system. As a first step, we used the two-hybrids assay to localize a minimal Rad53-interacting domain (Rad9-MID). The smallest deletion mutant tested that has full binding activity contains Rad9 amino acids 542-620. Since the working hypothesis is that Rad53 FHA2 directly recognizes phospho-Rad9, and that the Rad9 kinase is Mec1, we examined the Rad9-MID for sequences characteristic of Atm family protein kinase phosphorylation sites. DNA PK preferentially phosphorylates SQ/TQ sequences, especially in an acidic environment [2, 3]. The Atm sequence specificity seems similar, since two apparent *in vivo* targets for Atm, p53 and Abl, are both phosphorylated on sites that fit these criteria. This suggests that Mec1 would also share this target specificity. Overall, Rad9 contains 9 SQ or TQ sites, of which only one is contained within the Rad9-MID. Remarkably, this site is embedded within an acidic peptide and is nearly identical to a site on p53 phosphorylated by Atm (LSQE versus LTQE for Rad9) [4, 5].



542 RDDIIIAGS SDFNEQKEIT DRIYLQLSGK QISDSGSDET ERMSPNELDT KKESTIMSEV
ELTQELPEVE EQQDLQTSFK 620

A site-directed mutation of the Rad9-MID replacing Rad9 T603 with A almost completely inhibited interaction in the 2 hybrids assay, even though the level of expression of the fusion protein was similar to its non-mutated counterpart. We next determined whether the Rad9-MID is a substrate for DNA PK. GST-Rad9-MID was expressed in bacteria, purified on glutathione-agarose, cleaved from the GST tag with thrombin, and incubated with DNA PK. DNA PK phosphorylated RAD9-MID well, but failed to phosphorylate the same protein with the T603 to A substitution, despite the presence of 14 other serine and threonine residues on the same peptide. Hence these data show that T603 is required for interaction of RAD9 with Rad53, and that it is an in vitro target for a protein kinase related to Mec1.

II. Technical Objective 3. Screening based upon protein sequence homology.

We proposed to use a nested PCR strategy to identify mammalian homolog(s) of the checkpoint protein kinase Rad53. This gene, and its homologs in fission yeast and *Drosophila melanogaster*, are characterized by the linkage of an FHA domain to a protein kinase domain. Briefly, the approach was to produce a pool of cDNAs by PCR amplification of a Jurkat cell cDNA library using a degenerate upstream primer that would recognize FHA domains, and a degenerate downstream primer that would recognize protein kinase domains. The pool was then enriched for PCR products that fall within the expected 500-1200 b.p. size range, and cloned into Bluescript SKII+. Plasmids with the appropriate-sized inserts were identified, and representative members were analyzed by nucleotide sequence and DNA database searches.

The detailed procedure and results are shown on the following page. Thus far, only a single protein kinase gene, *CHK1* has been identified. This result is intriguing, because mammalian *CHK1* had already been identified on the basis of homology to *RAD53*. However, with the clones that have been sequenced to date, it appears that priming by the degenerate FHA domain primers was non-specific. This is not a complete surprise, since the degeneracy of FHA domains and the punctate nature of conserved areas has made designing primers a major challenge. (However, the primers chosen did correctly amplify RAD53 in control experiments). Sequencing and characterization of additional clones is still underway.

Summary of PCR Screen for FHA Protein Kinase

Step

Goal

- | | |
|--|---|
| 1 primer design
2 reaction optimization

3 prepare initial template

4 Reaction 1
5 purify DNA
6 Reaction 2 | design degenerate FHA and Kinase domain primers
determine anneal temps for the all reactions
optimize reaction to reduce background
test against specific and nonspecific/dummy DNA
amplify (liquid stock) lambda gt11 library
(resting Jurkat library from S. Weissman)
prepare clean DNA from phage
amplify kinase domain-containing sequences
remove extra primers, primer dimers
amplify kinase domain-containing sequences that also contain an upstream domain |
|--|---|

FHA	DKD		
	1	2	3
1	A	B	C
2	D	E	F
3	G	H	I

- | | |
|--|---|
| 7 size select DNA via agarose gel

8 clone inserts
9 size inserts of transformants
by uncut size
by insert PCR | enrich for inserts of expected size
selected ~500-1200 bp range
clone PCR products into EcoRV cut, CIP'd pBluescriptSKII+
identify non-vector-only transformants by uncut size (by comparison)
PCR insert (via T3/T7) to size
select 2 of each pool for sequencing based on expected and observed size |
|--|---|

Results

major detected homology

A	vector
B	CHK1
C	APEX
D	appears identical to C2, but in opposite orientation
E	TRIP2
F	unknown
G	identical to E1
H	snurportin1
I	CHK1

III. Technical Objective 3A. Identification of authentic mammalian Rad53/Cds1

Another approach that we had proposed to use (Technical Objective 3A) was to further characterize ESTs related to *RAD53* by sequencing homologous cDNAs. In the course of this work, other research groups used this strategy to identify a mammalian homolog to the *RAD53*-related yeast protein kinase *DUN1*. This gene, denoted *CHK2* or *hCDS1* [6-8], turned out to be a better homolog of *RAD53* than of *DUN1*. As we predicted, this gene encodes an FHA-containing protein kinase. It is regulated by both DNA replication and DNA damage checkpoints, and its regulation in response to ionizing radiation requires functional Atm. We now have a cDNA clone on hand in the laboratory that encodes full-length Chk2/Cds1. This means that a major prediction and goal of the project has now been fulfilled.

(7) RESEARCH ACCOMPLISHMENTS

- Expression of functional Mec1, and identification of protein kinase activity.
- Identification of a domain on Rad9 that is required for interaction with Rad53
- Identification of a putative phosphorylation target site for Atm family members that is likely to be the target for the Rad53 FHA domain
- Completion of first round screens for FHA-containing protein kinase genes

(8) REPORTABLE OUTCOMES

none

(9) CONCLUSIONS

Alterations in DNA damage response pathways, including checkpoint pathways, are known to occur frequently in human carcinogenesis. Such changes in breast cancer include mutations in p53, Brca1, and Brca2. Genetic analysis of yeasts has identified DNA checkpoint pathways that are necessary to maintain the genome in the face of DNA damage. The identification of the human ATM gene, which is homologous to a major element in yeast checkpoint pathways, led to the hypothesis that other member of these checkpoint pathways would also be conserved in mammals. The purpose of the research that we are conducting has been to, first, identify such human homologs, and second, determine how they function in mammalian checkpoint pathways. Our work in this first year of the research has laid the groundwork for identification of proteins that are functionally related to the checkpoint protein Rad9. This will make it possible in the second year to move to screening for Rad9-related mammalian genes, which are yet to be identified. Since Mec1/Rad9/Rad53 interactions are a prototype for an important machine in the checkpoint control apparatus, our work this year to identify the Rad53 interaction domain of Rad9 represents a major step forward in understanding of this machinery. Moreover, the Rad9 site represents the first identified phosphopeptide that interacts with a FHA domain. This phosphopeptide/FHA interaction is itself the paradigm for function of all FHA domains, and represents a major advance in understanding signal transduction by these modules.

A second goal this year was to identify mammalian homologs of the protein kinase Rad53. We began by using a PCR screen based upon the protein structures (FHA domain and protein kinase domain) that would be contained in such a homolog. This screen has thus far been unsuccessful in identifying a homolog, although there is still residual sequencing to be performed. However, other laboratories have now identified the protein kinase gene that we sought. We now have the clone encoding this gene, and a major challenge will be to characterize the function of mammalian Rad53 (Chk2/Cds1), as originally described in Technical Objective 4.

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